PHYSIOLOGY AND REPRODUCTION

Detection of *Campylobacter* or *Salmonella* in Turkey Semen and the Ability of Poultry Semen Extenders to Reduce Their Concentrations[1,2]

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ABSTRACT *Campylobacter* and *Salmonella* are the most commonly reported pathogens causing foodborne illness in the United States. In turkeys, the potential that semen used for artificial insemination is contaminated with these foodborne pathogens has not been investigated. Because semen on turkey farms is pooled and then used to inseminate multiple hens, contaminated semen could easily spread these bacteria throughout entire flocks via artificial insemination. The objectives of this study were to 1) determine if semen from commercial turkey farms contained these foodborne pathogens and 2) if present, evaluate the efficacy of semen extenders to reduce or eliminate *Campylobacter* and *Salmonella* from semen. Semen was collected from randomized pools of ejaculates from 10 to 30 toms per farm from 6 flocks over a 7-wk period and, on occasion, was found to contain *Campylobacter*, *Salmonella*, or both. To evaluate the efficacy of semen extenders to reduce or eliminate pathogens, pooled ejaculates were challenged with *Campylobacter* or *Salmonella* and treated with commercial poultry extenders containing various concentrations of antibiotics or an antibiotic combination previously demonstrated to remove *Campylobacter* from mammalian semen. Results demonstrate that commercial turkey semen may contain *Campylobacter* or *Salmonella*, and the semen extenders tested either did not reduce the bacteria or reduced but did not eliminate these bacteria from semen. We concluded that semen may be a potential vehicle for *Campylobacter* transfer to hens, and, if this is true, development of a method for eliminating pathogens in semen before insemination could reduce the risk of colonization.

(*Key words*: *Campylobacter*, *Salmonella*, semen extender, turkey)

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INTRODUCTION

Campylobacter and *Salmonella* are the most commonly reported pathogens causing foodborne infections in the United States (CDC, 2002) with an estimated 2.1 to 2.4 million cases reported annually (Altekruse et al., 1999). Epidemiological evidence has implicated raw poultry products as a significant source of human infection (Blaser, 1997; Todd, 1997; Daniels et al., 2002; Thorns, 2002), and several investigators have reported a high proportion of *Campylobacter* and *Salmonella* contamination in retail chicken and turkey carcasses (Norkrans and Svedhem, 1982; Genigeorgis et al., 1986, Shane, 1992; Stern and Line, 1992; Corry and Attabay, 2001; Zhao et al., 2001; Logue et al., 2003; Fratamico, 2003).

Foodborne pathogens such as *Campylobacter* seldom cause apparent clinical disease in poultry flocks (Hargis et al., 2001; Newell and Fearnley, 2003). Therefore, the link between semen contamination and transmission to the hen may occur without apparent observable signs or alterations in fertility. *Salmonella* can be vertically transferred from parent flocks to progeny through the transovarian route (Baker et al., 1980; McGarr et al., 1980; Guthrie 1992; Notermans et al., 1992; Ranta and Maijala, 2002). Reiber and colleagues (1995) reported that chicken semen might serve as one of the mechanisms of transmission of *Salmonella* to hens and eggs. Available evidence also supports the idea that *Campylobacter* may be able to trasverse the reproductive tract of chickens and contaminate the fertile egg and subsequent offspring (Cox et al., 2002a). These researchers reported that the same clonal isolates from an infected broiler breeder flock also infected their offspring, although they were never in direct

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Abbreviation Key: ATCC = American Type Culture Collection; BPD = Butterfield's phosphate diluent; API = analytical profile index.

contact with each other and were housed 20 miles apart. To our knowledge, the potential for semen contamination and transmission in the turkey has not been investigated. Semen collection, by nature of the tom's anatomy, is predisposed to fecal contamination (Donoghue, 1997), which may include *Campylobacter* or *Salmonella* (Thorns, 2002; Newell and Fearnley, 2003). The practice of pooling semen from large numbers of toms, which is the standard procedure in the industry (Donoghue, 1999), could potentially increase the possibility of contaminating multiple flocks. Additionally, because turkey semen has not been considered a source for contamination, antibacterial extenders have not been developed, tested, marketed, or targeted specifically for efficacy against human foodborne pathogens such as *Campylobacter* or *Salmonella*. Therefore, the objectives of this study were to 1) evaluate turkey semen collected randomly from commercial turkey farms and determine whether *Campylobacter* or *Salmonella* was present in the semen and 2) evaluate the efficacy of commercially available semen extenders and other antibiotic combinations to reduce or eliminate *Campylobacter* and *Salmonella* from semen.

MATERIALS AND METHODS

Study 1. Evidence of Campylobacter and Salmonella in Commercial Turkey Semen

Six commercial turkey flocks were evaluated for the presence of bacteria over a 7-wk period. Semen containing a randomized pool of ejaculates from 10 to 30 toms per farm were evaluated for endogenous bacterial counts and identification of specific pathogens using standard bacteriological methods with modifications (Corrier et al., 1999; Cox et al., 2002b). Briefly, for total bacterial enumeration, 100 uL of semen was added to 9.9 mL of Butterfield's phosphate diluent (BPD, Andrews et al., 1978), 10-fold serial dilutions in BPD were performed, and then 100 uL of each dilution was plated on tryptic soy agar[4] plates. Plates were incubated aerobically at 37°C overnight. Colonies on each plate were then counted on a Leico Darkfield Plate Colony Counter,[5] and the direct counts were converted to $\log_{10}$ colony-forming units per milliliter. Isolated colonies were identified using analytical profile index (API)[6] test strips. *Campylobacter* isolation was accomplished by pipetting 10 uL of raw semen onto Campycefex plates (Cox et al., 2002b); the plates were then incubated for 24 to 48 h at 42°C in a microaerophilic environment (5% O_2, 10% CO_2, and 85% N_2). After incubation,

characteristic colonies were confirmed as *Campylobacter* by observation of typical cellular morphology with a phase contrast microscope, a commercial latex agglutination kit specific for *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter laridis*,[7] API[6] Campy test strips, and PCR (see procedure below). *Salmonella* was isolated by preenrichment of semen in tetrathionate broth[8] overnight at 42°C and then plated on brilliant green agar (BGA).[9] Pink colonies typical of *Salmonella* were checked for agglutination of *Salmonella* antisera Groups A-I and specifically Group D. *Salmonella* was verified with API[6] 20E test strips.

To further identify *Campylobacter* or *Salmonella* isolates, DNA was extracted from semen samples for use in a PCR method as described by Soumet et al. (1999) and a sample enrichment protocol described previously (Gonzalez et al., 1997). PCR reactions were performed according to Vanniasinkam et al. (1999) with an annealing temperature of 63°C.

Study 2. Evaluating the Efficacy of Commercial Semen Extenders to Reduce or Eliminate Campylobacter and Salmonella in Turkey Semen

To evaluate the efficacy of commercially available semen extenders to reduce or eliminate *Campylobacter* and *Salmonella* from semen, pooled ejaculates from 10 to 30 toms were aliquoted into 5 semen extender treatments: extender 1: without antibiotic (Field Ready Turkey Extender Green[10]); extender 2: with 200 μ/mL of gentamicin (Field Ready Turkey Extender Blue[10]); extender 3: with 2.5 ug/mL gentamicin and 8.8 ug/mL tylosin (Ovodyl[10]); extender 4: TSS medium[10] (a poultry semen extender containing a nondisclosed quantity of enrofloxacin); and extender 5: a cocktail of 250 μmL gentamicin, 200 μmL tylosin, 150 μmL lincomycin, 1,000 μmL kanamycin, and 300 μmL spectinomycin diluted into extender 1. Extenders 1 to 4 were commercially available turkey semen extenders. Extender 5 was a combination of antibiotics recommended by the Certified Semen Services of the National Breeders Association[11] (Shin et al., 1988) with modifications in antibiotic concentrations (Huyser et al., 2004). This combination of antibiotics was effective in eliminating *Campylobacter fetus subsp. venerealis* in bovine semen and was not detrimental to fertility in cattle (Lorton et al., 1988; Shin et al., 1988). Semen was diluted with extender 1:1(vol/vol). Prior to addition of the extenders, semen was unchallenged (control) or was challenged with *Campylobacter*, American Type Tissue Culture (ATCC) strain 33291, or a wild-type *Salmonella enteritidis*, averaging approximately 2.9×10^6 for *Campylobacter* and 1.9×10^8 cells/mL for *Salmonella*. To mimic industry practice prior to insemination, semen treatments were incubated at room temperature for 30 min and then evaluated for bacterial counts. Each sample was cultured for total bacterial count, *Campylobacter*, or *Salmonella* with the procedures described in study 1. For bacterial enumeration, 10-fold serial dilutions in BPD were performed for each

TABLE 1. The ability of semen extenders containing various antibiotics to reduce native bacterial concentrations, or challenged *Salmonella* or *Campylobacter* concentrations (cfu/mL) in commercial turkey semen (mean of 3 separate trials)[1,2]

Treatment[3]	Antibiotic	Total bacterial counts	Challenge[1]	
			Salmonella	*Campylobacter*
Extender 1	none	$7.5 \times 10^{5,a}$	$1.1 \times 10^{8,b}$	$3.0 \times 10^{6,b}$
Extender 2	Gentamycin	$3.1 \times 10^{5,a}$	$1.2 \times 10^{7,ab}$	$2.0 \times 10^{6,b}$
Extender 3	Tylosin/Gentamycin	$8.6 \times 10^{5,a}$	$1.1 \times 10^{8,b}$	$2.6 \times 10^{6,b}$
Extender 4	Enrofloxacin	$8.1 \times 10^{5,a}$	$1.3 \times 10^{8,b}$	$2.3 \times 10^{6,b}$
Extender 5	Spectinomycin Tylosin/Gentamycin Lincomycin/Kanamycin	$3.0 \times 10^{5,a}$	$1.5 \times 10^{4,a}$	$2.6 \times 10^{5,a}$

[a,b]Means within the same column with no common superscript differ significantly ($P < 0.05$). All data were $\log_{10}$ transformed for statistical analysis. For ease of presentation, arithmetic means are presented.

[1]Pooled semen from commercial turkey farms was co-incubated with extender treatments for 30 min at 23°C prior to bacterial determination in 3 separate trials. Average native bacterial counts was 1.8×10^6 cfu/mL prior to addition of extenders. Immediately before addition of extenders, a wild type *Salmonella enteriditis* or *Campylobacter* (ATCC 33291) isolates were added to semen averaging 1.9×10^8 or 2.9×10^6 cells/mL, respectively.

[2]Total bacterial counts, *Salmonella* or *Campylobacter* concentrations (cfu/mL) were determined after the 30 min co-incubation with semen extenders using standard microbiological procedures (see M&M section for details).

[3]Extenders 1–4 are commercially available turkey semen extenders. Extender 5 is a combination of antibiotics recommended by the Certified Semen Services of the National Breeders Association with modifications (see M&M section for details).

sample, and then 100 uL of each dilution was plated on tryptic soy agar (total bacterial count), *Campylobacter*-Line agar (*Campylobacter* counts), or brilliant green agar plates (*Salmonella* counts). Three separate trials were conducted.

Study 3. Evaluating the Efficacy of Semen Extenders on Turkey Semen Challenged with an ATCC strain of Campylobacter or Wild-Type Campylobacter Isolates

Our third study was conducted to evaluate whether the antibiotic cocktail demonstrating the greatest in vitro efficacy in study 2, extender 5, was as effective against 3 wild-type *Campylobacters* as it was against an ATCC laboratory strain (33291) of *Campylobacter*. The 3 wild-type isolates were previously collected from turkey semen (8144 and 8214) or turkey ceca (8314). Three separate trials were conducted using the procedures described in study 2. The average concentrations of *Campylobacter* isolates used for the in vitro challenge were 3.3×10^6, 4.4×10^7, 2.4×10^7, or 5.6×10^6 cells/mL for the ATCC (33291), 8144, 8214, or 8314 strain, respectively.

Statistical Analysis

Data were analyzed by ANOVA using the Statistical Analysis System (SAS, 1994) GLM program. Endogenous bacteria, *Campylobacter* colonies, and *Salmonella* colonies were logarithmically transformed ($\log_{10}$ cfu) prior to analysis to achieve homogeneity of variance (Byrd et al., 2003). Treatment means were partitioned by least squares means analysis (SAS, 1994). A probability of $P < 0.05$ was required for statistical significance. For ease of presentation, some data were presented as arithmetic means.

RESULTS

Study 1. Evidence of Campylobacter and Salmonella in Turkey Semen

With morphological evaluation and latex agglutination methods *Campylobacter* was detected in semen from 2 commercial turkey flocks tracked for 7 wk (August to September 2002). *Campylobacter* was found on 1 occasion on 1 farm and 6 of the 7 wk on a second farm. Samples determined to be positive for *Campylobacter* were further identified as *C. coli* by PCR. *Salmonella enteritidis* was also identified by PCR, by using genus species-specific primers, on 1 occasion in 2 of the 7 flocks tracked. *Pseudomona aeruginosa*, *Escherichia coli*, *Escherichia hermannii*, *Staphylococcus lentus*, *Staphylococcus sciuri*, and *Staphylococcus capitis* were also identified in turkey semen.

Study 2. Evaluating the Efficacy of Commercial Semen Extenders to Reduce or Eliminate Campylobacter and Salmonella in Turkey Semen

Total bacterial counts were not reduced in semen by using any extenders with antibiotics vs. an extender without antibiotics (control) after a 30-min incubation (Table 1). After in vitro challenge, neither *Salmonella* or *Campylobacter* concentrations were reduced with the use of the commercial extenders 2, 3 or 4 compared with a control extender without antibiotic (extender 1, Table 1). For extender 5, *Salmonella* and *Campylobacter* concentrations were reduced after a 30-min incubation period compared with extender 1 without antibiotic (Table 1).

TABLE 2. The ability of a semen extender without antibiotics (Extender 1) versus an extender with multiple antibiotics (Extender 5) to reduce *Campylobacter* concentrations (cfu/mL) in commercial turkey semen after addition of an American Type Tissue Culture (ATCC) or three wild type *Campylobacter* isolates (mean of 3 separate trials)[1,2]

| | | | *Campylobacter* challenge[1] | | |
| | | | Wild-type *Campylobacter* | | |
Treatment[3]	Endogenous *Campylobacter*	*Campylobacter* ATCC (33291)	8144	8214	8314
Extender 1	$4.1 \times 10^{2,b}$	$4.9 \times 10^{6,b}$	$3.4 \times 10^{7,b}$	$9.1 \times 10^{6,b}$	$8.5 \times 10^{4,b}$
Extender 5	$1.2 \times 10^{1,a}$	$5.8 \times 10^{5,a}$	$4.9 \times 10^{5,a}$	$2.4 \times 10^{3,a}$	$1.2 \times 10^{3,a}$
Differences between Extender 1 and 5 (Log_{10} $\bar{x} \pm$ SEM)					
	0.74 ± 0.6^{x}	1.2 ± 0.3^{x}	2.7 ± 0.8^{xy}	4.4 ± 1.0^{y}	2.6 ± 0.9^{xy}

[a,b]Means within the same column with no common superscript differ significantly ($P < 0.05$). All data were $\log_{10}$ transformed for statistical analysis. For ease of presentation, arithmetic means are presented.

[x,y]Means within the same row with no common superscript differ significantly ($P < 0.05$). All data were $\log_{10}$ transformed for statistical analysis.

[1]Pooled semen from commercial turkey farms was co-incubated with extender treatments for 30 min at 23°C prior to *Campylobacter* determination in 3 separate trials. Immediately before addition of extenders, the four *Campylobacter* strains was added to semen averaging 3.3×10^6, 4.43×10^7, 2.4×10^7 or 5.6×10^6 cells/mL for the ATCC (strain 33291), 8144, 8214 or 8314 isolates, respectively.

[2]*Campylobacter* concentrations (cfu/mL) were determined after the 30 min co-incubation with semen extenders using standard microbiological procedures (see M&M section for details).

[3]Extender 1 is a commercial turkey extender without antibiotic (Field Ready Turkey Extender Green), Extender 5 is a combination of antibiotics recommended by the Certified Semen Services of the National Breeders Association with modifications (see M&M section for details).

Study 3. Evaluating the Efficacy of a Selected Semen Extender (Extender 5) on Turkey Semen Challenged with an ATCC Campylobacter or Wild-Type Campylobacter Isolates

Extender 5 reduced ($P < 0.05$) *Campylobacter* concentrations for the ATCC and wild-type *Campylobacter* isolates when compared with the control extender, extender 1, without antibiotics (Table 2). Endogenous *Campylobacter* concentrations were also reduced by the use of extender 5. However, endogenous *Campylobacter* concentrations were near or below the lower limits of detection for *Campylobacter* (log 10^1cfu/mL). Therefore, it is difficult to accurately measure differences bordering on the method's detection limit. The highest endogenous *Campylobacter* concentration detected was 1.2×10^3 cfu/mL. To evaluate whether extender 5 had a greater effect on any of the isolates after in vitro challenge, the relative difference between extenders 1 and 5 were determined (Table 2). When exposed to extender 5, there was a greater reduction for only 1 of 3 wild-type *Campylobacter* isolates (isolate 8214) when compared with the ATCC *Campylobacter* strain.

DISCUSSION

We detected *Campylobacter* and *Salmonella* in turkey semen collected from commercial farms. Because turkey semen has not been considered a source for these bacteria, antibacterial extenders have not been previously developed or tested for efficacy against human foodborne pathogens. In the early 1970s, *Mycoplasma meleagris* was determined to be transmittable through semen. Due to its detrimental influence on the reproductive productivity of turkeys, methods were developed to eliminate the contaminant from semen (Saif and Brown, 1972). The effect of *Campylobacter* or *Salmonella* contamination in semen on reproductive performance, however, has not been determined. Because *Campylobacter* and *Salmonella* seldom cause apparent clinical disease in poultry flocks (Hargis et al., 2001; Newell and Fearnley, 2003) the effects of these pathogens on fertility or hatchability have not been evaluated. However, as the turkey industry works to reduce foodborne pathogens in turkey products, determining and reducing sources of contamination are important human food safety considerations.

Reiber and colleagues (1995) demonstrated that rooster semen might serve as the vehicle of transmission of *Salmonella* to the hen and eggs. *Campylobacter* has also been found in semen and vas deferens of commercial roosters (Cox et al., 2002b). In additional studies involving broiler breeder hens, *Campylobacter* was readily isolated from all sections of the hen's reproductive tract (Buhr et al., 2002). Recently, *Campylobacter* has also been isolated throughout the reproductive tracts of turkey toms and hens (Cole et al., 2004).

Semen collection, due to anatomy of toms, is predisposed to fecal contamination. Bacterial contamination is prevalent in poultry semen with reports of an average of 2.2 million bacteria per milliliter in chickens (Wilcox and Shorb, 1958) and 1.3 billion bacteria per milliliter in turkey semen (Gale and Brown, 1961). The most frequently isolated genera included *Escherichia*, *Staphylococcus*, *Bacillus*, and *Enterococcus*. Practitioners are cautioned to remove visible debris from semen; however, fertility levels of >90% can be maintained without filtering or centrifuging out contaminants in turkey semen. Although many farms filter semen prior to insemination to remove urates and fecal matter (Grimes et al., 1997), in the current study

we found *Campylobacter* and *Salmonella* in filtered and nonfiltered semen provided to us by commercial turkey farms (personal observation, data not shown).

Our results indicate that the commercially available poultry semen extenders tested were not consistent in reducing *Campylobacter* or *Salmonella* concentrations after in vitro challenge. Only the cocktail of antibiotics in extender 5 was effective against *Campylobacter* and *Salmonella* (Table 1) or against the wild-type *Campylobacter*s previously isolated from turkey semen or ceca (Table 2). Sexton et al. (1980) reported on an extensive evaluation of 40 antibiotics for efficacy against total bacterial growth and the effects of these antibiotics on subsequent fertilizability of sperm after in vitro storage of rooster semen. Only 4 of the forty antibiotics in their study were effective at reducing bacterial content without negatively influencing fertility, which included gentamicin at 250 μmL. Studies by Saif and Brown (1972) have demonstrated that 200 μmL of gentamicin is effective against *Mycoplasma melagridis*. Therefore gentamicin has become the antibiotic of choice for poultry semen extenders sold commercially (Sexton et al., 1980). Two of the 4 antibiotics (gentamicin and kanamycin) tested in the Sexton study were present in our extender 5. The other 3 antibiotics in this cocktail, spectinomycin, tylosin, and lincomycin, have not been evaluated for their effects on fertility in poultry. In previous work attempting to eliminate bacterial growth in turkey semen, concentrations of antibiotics capable of eliminating all bacterial growth were detrimental to fertility (Donoghue, unpublished data). The antibiotic combination in extender 5 is not detrimental to bull sperm fertilizing ability (Lorton et al., 1988) or human (Huyser et al., 2004) or turkey (data not shown) sperm viability in vitro. As extenders are formulated to eliminate *Campylobacter* or *Salmonella* from turkey semen, antibiotic source and concentration will need to be tested to insure they are not harmful to sperm viability and function.

It was not the intention of this study to do a comprehensive survey of the concentrations of *Campylobacter* or *Salmonella* in commercial turkey semen. It is probable that there is variation due to semen collection technique or fecal contamination. In addition, variation in semen samples could be due to farm location and seasonality of these bacteria, as, for example, has been demonstrated for overall flock *Campylobacter* concentrations (see review, Newell and Fearnley, 2003). The concentrations of *Campylobacter* and *Salmonella* used for the in vitro challenge are probably much higher than normally found in semen. The maximum detection of 1.2×10^3 cfu/mL of endogenous *Campylobacter* in semen samples supports this idea. Unfortunately, the use of a challenge dose that may be closer to the concentrations found in semen would approach the detection limit of the method used in this study (1 log) and make it difficult to obtain accurate results. It may be that extender 5 would eliminate *Campylobacter* or *Salmonella* from turkey semen if these bacteria do occur at lower concentrations; however, as stated above, the effect of this combination on subsequent fertility would have to be determined.

The issue of antimicrobial resistance should be considered when developing semen extenders with antibiotics for the reduction or elimination of *Campylobacter* or *Salmonella*. Recent evaluation of *Campylobacter* (Ge et al., 2003) and *Salmonella* (Chen et al., 2004) isolates collected from retail meats, including turkeys, indicates several isolates are resistant to 1 or more antibiotics. The enhanced ability of extender 5 to reduce only 1 out of 3 wild-type *Campylobacter* isolates (Table 2) probably relates to its sensitivity (reduced resistance) to these antibiotics. Alternatives without antibiotics may be more successful for removing these bacteria from semen. One of our strategies to reduce *Campylobacter* concentrations in semen is to alter the environmental conditions (Cole et al., 2004) or the chemical make up of the extenders.

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